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Response of the leaf photosynthetic rate to available nitrogen in erect panicle-type rice (*Oryza sativa* L.) cultivar, Shennong265

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ABSTRACT

Increasing the yield of rice per unit area is important because of the demand from the growing human population in Asia. A group of varieties called erect panicle-type rice (EP) achieves very high yields under conditions of high nitrogen availability. Little is known, however, regarding the leaf photosynthetic capacity of EP, which may be one of the physiological causes of high yield. We analyzed the factors contributing to leaf photosynthetic rate (P_n) and leaf mesophyll anatomy of Nipponbare, Takanari, and Shennong265 (a EP type rice cultivar) varieties subjected to different nitrogen treatments. In the field experiment, P_n of Shennong265 was $33.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the high-N treatment, and was higher than that of the other two cultivars because of its high leaf nitrogen content (LNC) and a large number of mesophyll cells between the small vascular bundles per unit length. In Takanari, the relatively high value of P_n ($31.5 \mu\text{mol m}^{-2} \text{s}^{-1}$) was caused by the high stomatal conductance (g_s ; $.72 \text{ mol m}^{-2} \text{s}^{-1}$) in the high-N treatment. In the pot experiment, the ratio of P_n/C_i to LNC, which may reflect mesophyll conductance (g_m), was 20–30% higher in Nipponbare than in Takanari or Shennong265 in the high N availability treatment. The photosynthetic performance of Shennong265 might be improved by introducing the greater ratio of P_n/C_i to LNC found in Nipponbare and greater stomatal conductance found in Takanari.

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Introduction

Food shortage is a growing threat in light of continued population growth and increasing competition between food and energy for arable land. One of the most important cereal crops, rice (*Oryza sativa* L.), is widely cultivated, especially in Asian countries. It constitutes a major source of carbohydrates for more than half of the world's population (<http://www.fao.org/docrep/018/i3107e/i3107e03.pdf>). A group of high-yielding *japonica* rice cultivars, characterized by an erect panicle (EP), and leaves are expected to feed the growing population and were released as commercial varieties in China. Recently, EP varieties were cultivated on 1.3 million ha throughout China (Song et al., 2013). According to the previous studies, EP varieties achieve very high yields under conditions of high nitrogen availability (Chen et al., 2007; Li, 2003; Zhang et al., 2002), and some genes related to the high yield of EP varieties have been detected (Huang et al., 2009; Wang et al., 2009; Zhu et al., 2009). Little is known, however, regarding the leaf photosynthetic capacity of EP, in spite of its importance in determining the yield of rice.

In general, leaf nitrogen content (LNC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)

content are factors that can potentially limit crop production and leaf photosynthesis (Makino et al., 1983; Sinclair & Horié, 1989). Besides, short and dark colored leaves have been observed in EP varieties (Lv & Cheng, 2010; Zhu et al., 2009), and these leaves may be related either to high LNC or to unique leaf anatomy. We hypothesized that leaves of EP cultivars have a greater leaf photosynthetic capacity under nitrogen-rich treatments and the capacity is sustained by unique mechanisms, which may include leaf morphology and mesophyll structure. To test this hypothesis, Shennong265 (a typical EP rice) was grown under different nitrogen fertilizer treatments, along with Nipponbare (a representative *japonica* rice) and Takanari (a high-yielding *japonica-indica* cross-variety). The response of P_n to the nitrogen treatments and its physiological or anatomical basis was examined for Nipponbare, Takanari, and Shennong265.

Materials and methods

Plant materials and growing conditions

For the field experiment, Nipponbare, Takanari, and Shennong265 were grown in a paddy field at the experimental

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**Table 1.** The total amount of N fertilizer applied for the pot and field experiments.

	Pot experiment (g pot ⁻¹)	Field experiment (g m ⁻²)
Low-N	.5	3
Standard-N	1.2	Not applicable
High-N	2.1	17

farm of the Graduate School of Agriculture, Kyoto University (35° 2' N, 135° 47' E; altitude 65 m above sea level). Seeds of each cultivar were sown on 7 May and transplanted on 6 June 2013 to the paddy field in alluvial loam soil classified as Haplaquept. The size of each plot was > 12 m², and hill spacing was .15 × .30 m (density: 22.2 hills m⁻¹) with one plant per hill. The randomized block design was established with two replications. For the low-nitrogen treatment, Eco-long (JCAM AGRI), a slow release fertilizer, was applied at rates of 3.00, 2.36, and 2.79 g m⁻¹ for N, P₂O₅, and K₂O, respectively. The same fertilizer was applied at rates of 12.00, 9.43, and 11.14 g m⁻¹ for N, P₂O₅, and K₂O, respectively, for the high-nitrogen treatment. Additionally, 5 g m⁻¹ of LP cote (JCAM AGRI), a coated nitrogen fertilizer, was applied to the high-nitrogen treatment group as a basal fertilizer (Table 1).

For the pot experiment, seeds of the same varieties were sown on 7 May and transplanted on 6 June 2013 into 3.8-L pots filled with soil from the field (alluvial loam soil) at a density of one plant per pot with five replicates. Ammonium sulfate (Sumitomo Chemical), monocalcium phosphate (Taki Chemical), and potassium chloride (Mitsubishi) were applied at rates of .5–.5–.5 g per pot (N–P₂O₅–K₂O) as the basal fertilizer, respectively. For standard N and high N treatments, additional N fertilizer (ammonium sulfate) for the standard N and high N treatments was applied every two weeks to total nitrogen supplies to 1.2, and 2.1 g, respectively (Table 1).

Leaf photosynthetic rate and stomatal conductance

The light-saturated net photosynthetic rate (P_n) and the stomatal conductance (g_s) of the uppermost, fully expanded leaves of each variety were measured with a portable gas exchange system (Li-6,400; Li-COR). The measurements were conducted from 10:00 to 13:00 when the plants were at the active tillering stage under natural sunlight and light-saturated conditions on 16 and 17 July 2013, for the pot and field experiments, respectively. The reference and sample CO₂ in the leaf chamber was 380 ± 2 and 359 ± 12 μmol mol⁻¹, respectively, for all measurements. The photosynthetic photon flux density (PPFD) was 2,000 μmol m⁻² s⁻¹ with 10% blue light. The block temperature was 30 °C, and the observed leaf temperature was 30.6 ± 1.0 °C for all the measurements. The relative humidity in the leaf chamber ranged from 57.7 to 72.1%.

Measurement of leaf area index

In the field experiment, the aboveground parts of four plants per plot were harvested at the panicle initiation and heading stages. Shennong265, Takanari, and Nipponbare reached the panicle initiation stage on 9, 20, and 25 July 2013, respectively, and reached the heading stage on 1, 13, and 16 August 2013, respectively. Based on the number of tillers per plant, 3 samples were taken in total from 12 plants in each plot to balance the rice canopy. Green leaf blades were separated from the plant, and leaf area was measured with a portable leaf area meter (Li-3,080; Li-COR). The leaf area index (LAI) was calculated by dividing the measured leaf area by the planted area.

Quantitation of LNC

In both field and pot experiments, the leaves were collected after the measurement of P_n , frozen immediately with liquid nitrogen, and stored at –80 °C until use. The area of each leaf was determined with the portable leaf area meter. After the measurement of area, leaves were oven-dried at 80 °C for a minimum of 72 h and weighed to determine dry weight. Leaf mass per area (LMA) was calculated from single-leaf area and dry weight. Nitrogen concentration was quantified by the indophenol method after Kjeldahl digestion (Kjeldahl, 1883). LNC was calculated by multiplying LMA by nitrogen concentration. For LNC and LMA, four samples per plot were collected and then the values were averaged.

Analysis of leaf mesophyll anatomy

Samples for microscopic observation were taken from the uppermost, fully expanded leaves of plants grown in the pots. Cross sections of leaf mesophyll with 5-μm and 10-μm thicknesses were cut on a sliding microtome (REM-710; Yamato Kohki) and stained with 1% toluidine blue. The samples were observed with a light microscope (BHS-323; Olympus) at 200 × magnification. The microscopic images were recorded using a digital camera. The number of mesophyll cells between the two small vascular bundles (CN) was counted. The length between the small vascular bundles (L) was measured, and the cell number per unit area was calculated by dividing CN by L. The leaf thickness at the small vascular bundles was measured. Mean mesophyll cell area was determined by dividing the area occupied



Figure 1. The conceptual figure of mesophyll cell occupancy. Mesophyll cell occupancy was calculated as the ratio of the area enclosed by the dotted line to the area framed by the thick line (%). Tissues are bulliform cells (BF), small vascular bundle (SVB), epidermis (E), and mesophyll cells (M). The scale bar corresponds to 50 μm .

by the mesophyll cells (the dotted line in Figure 1) by CN. Mesophyll cell occupancy was calculated as the ratio of the area enclosed by the dotted line to the area framed by the thick line (%; Figure 1). These anatomical characteristics were determined with Image J software (NIH).

Statistical analyses

Statistical significance for differences among the three varieties per N treatment was determined by Tukey–Kramer multiple comparison test for all experiments. All statistical analyses were done with JMP v.11.2.0 software (SAS Institute). A two-way analysis of variance (ANOVA) was conducted to evaluate the effects of cultivar (C), nitrogen (N), and their interaction ($C \times N$) on P_n , g_s , P_n/C_i , and LNC for field experiment and on P_n , g_s , and P_n/C_i for pot experiments. An ANOVA was also used to detect differences in leaf anatomical characteristics among the three genotypes in the pot experiment. All of the two-way ANOVAs were conducted using Microsoft Excel.

Results

Photosynthetic capacity, LNC, and LAI in the field experiment

The value of P_n varied from 26.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for Nipponbare in the low-N treatment to 33.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for Shennong265 in the high-N treatment in the field experiment (Table 2). P_n of Shennong265 was the highest among the three cultivars under both N conditions. The value of g_s varied from .52 $\text{mol m}^{-2} \text{s}^{-1}$ for Nipponbare in the low-N treatment to .72 $\text{mol m}^{-2} \text{s}^{-1}$ for Takanari in the high-N treatment (Table 2). In both high and low-N treatments, the g_s of Takanari and Shennong265 was higher than Nipponbare. The P_n/C_i ratio varied from .088 $\mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol mol}^{-1}$ for Nipponbare in the low-N treatment to .114 $\mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol mol}^{-1}$ for Shennong265 in the high-N treatment (Table 2). In comparison with two other cultivars, Shennong265 exhibited high LNC values of 1.72 g m^{-2} and 1.63 g m^{-2} in the high-N and low-N treatments, respectively (Table 2). The effect of cultivars and N fertilizer levels on P_n , g_s , and P_n/C_i was significant ($p < .01$), whereas the interaction of these variables was not (Table 3). For the variation of LNC, the effect of cultivars and the interaction of cultivars and N fertilizer levels were significant ($p < .01$ and $p < .05$, respectively), whereas that of N fertilizer levels was not (Table 3). At the panicle initiation stage, LAI varied from .89 $\text{m}^2 \text{m}^{-2}$ for Shennong265 in the low-N treatment to 6.00 $\text{m}^2 \text{m}^{-2}$ for Nipponbare in the high-N treatment, respectively. At the heading stage, it varied from 1.84 $\text{m}^2 \text{m}^{-2}$ for Shennong265 in the low-N treatment to 6.63 $\text{m}^2 \text{m}^{-2}$ for Takanari in the high-N treatment, respectively (Figure 2). The LAI of Shennong265 was the lowest among the three cultivars under both N fertilizer levels. At panicle initiation stage, LAI of Shennong265 was only 36% and 30% against that of Nipponbare in low-N and high-N treatments, respectively. At heading stage, these values were 52% and 80%, respectively (Figure 2).

Table 2. The values of P_n , g_s , P_n/C_i at 380 mol mol^{-1} of reference CO_2 concentration, and LNC of Nipponbare, Takanari, and Shennong265 at different nitrogen fertilizer levels in the field experiment. Values are means \pm SD for $n = 2$ (LNC) and $n = 8$ (P_n , g_s , and P_n/C_i). Values followed by the same letters indicate no significant difference among cultivars or N treatments at $p < .05$ (Tukey's test).

		P_n ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		g_s ($\text{mol m}^{-2} \text{s}^{-1}$)		P_n/C_i ($\mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol mol}^{-1}$)		LNC (g m^{-2})	
Nipponbare	low	26.8 \pm 1.8	c	.52 \pm .06	d	.088 \pm .007	c	1.26 \pm .09	bc
	high	29.8 \pm 2.5	bc	.58 \pm .07	cd	.099 \pm .009	bc	.86 \pm .37	cd
Takanari	low	28.2 \pm 1.4	c	.59 \pm .03	cd	.091 \pm .005	bc	.64 \pm .04	d
	high	31.5 \pm 1.6	ab	.72 \pm .04	a	.102 \pm .006	ab	.99 \pm .13	cd
Shennong265	low	33.0 \pm 1.7	a	.62 \pm .06	bc	.111 \pm .007	a	1.63 \pm .06	ab
	high	33.8 \pm 3.1	a	.69 \pm .07	ab	.114 \pm .011	a	1.72 \pm .02	a

Table 3. F values and significance of cultivar (C), nitrogen fertilizer level (N), and their interactions (C × N) to P_n , g_s , P_n/C_i , LNC, and $P_n/C_i/LNC$ for the three cultivars in the field and pot experiments. A two-way analysis of variance (ANOVA) was conducted.

	F value	Probability	F value	Probability
P_n				
Cultivar (C)	28.6	<.01	2.3	NS
Nitrogen fertilizer level (N)	14.4	<.01	21.7	<.01
C × N	1.4	NS	1.5	NS
g_s				
Cultivar (C)	18.6	<.01	5.0	<.05
Nitrogen fertilizer level (N)	28.0	<.01	3.3	<.05
C × N	1.5	NS	.6	NS
P_n/C_i				
Cultivar (C)	32.1	<.01	3.1	NS
Nitrogen fertilizer level (N)	12.2	<.01	25.3	<.01
C × N	1.4	NS	2.1	NS
LNC				
Cultivar (C)	28.0	<.01	—	—
Nitrogen fertilizer level (N)	.0	NS	—	—
C × N	5.2	<.05	—	—
$P_n/C_i/LNC$				
Cultivar (C)	110.8	<.01	8.5	<.01
Nitrogen fertilizer level (N)	.8	NS	58.3	<.01
C × N	72.1	<.01	2.8	<.05

Notes. The total number of data points of field experiment except for LNC was 48 ($n = 48$), that of LNC was 12 ($n = 12$), and that of pot experiment was 45 ($n = 45$). NS indicates non-significance.

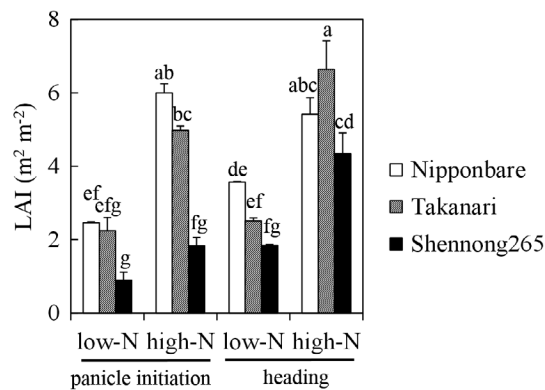


Figure 2. The values of LAI for Nipponbare, Takanari, and Shennong265 at different nitrogen fertilizer levels in the field experiment at panicle initiation stage and at heading stage. The error bars indicate the SD for $n = 2$. Columns with the same letters are not significantly different at $p < .05$ (Tukey's test).

Photosynthetic capacity, LNC, and leaf mesophyll anatomy in the pot experiment

The value of P_n ranged from $21.9 \mu\text{mol m}^{-2} \text{s}^{-1}$ for Nipponbare in the low-N treatment to $31.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ for Shennong265 in the high-N treatment in the pot experiment (Figure 3). P_n of Shennong265 was the highest among the three genotypes in two of the three N treatments. The effect of N fertilizer levels was significant ($p < .01$), whereas that of cultivars and the interaction of these variables was not (Table 3). The g_s of Takanari was the highest across all nitrogen treatments (Figure 3). The ratio of P_n/C_i ranged from $.075 \mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol mol}^{-1}$ for Nipponbare in the low-N availability treatment to $.112 \mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol mol}^{-1}$ for Shennong265 in the high-N treatment (Figure 3). The P_n/C_i of Shennong265 in the high-N treatment was 11% higher than that of Takanari

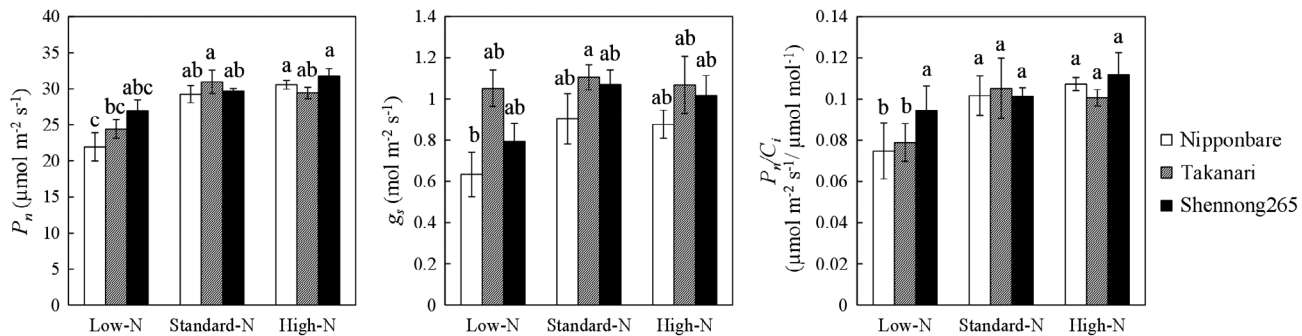


Figure 3. The values of P_n , g_s , and P_n/C_i of Nipponbare, Takanari, and Shennong265 at different nitrogen fertilizer levels at 380 mol mol^{-1} of reference CO_2 concentration in the pot experiment. Vertical bars represent the SD for $n = 5$. Columns with the same letters are not significantly different at $p < .05$ (Tukey's test).

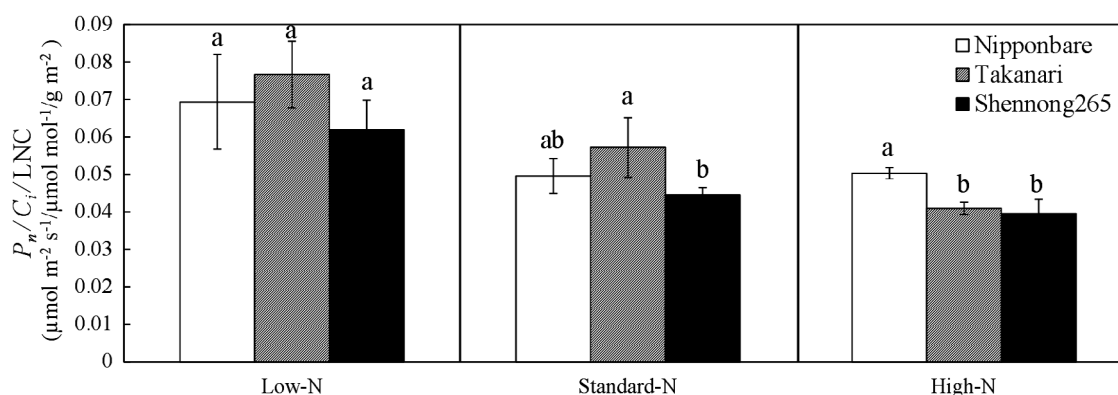


Figure 4. The values of $P_n/C_i/LNC$ of Nipponbare, Takanari, and Shennong265 at three different N fertilizer levels in the pot experiment. Error bars indicate SD for $n = 5$. Columns with the same letters are not significantly different at $p < .05$ (Tukey's test).

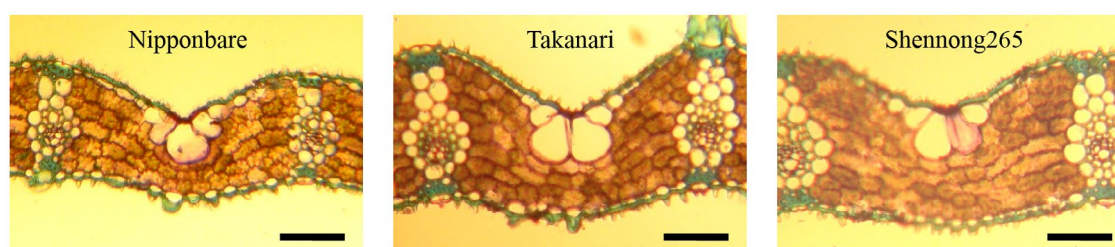


Figure 5. Cross sections of fully expanded leaves of Nipponbare, Takanari, and Shennong265. The scale bars in each figure correspond to 50 μm .

Table 4. LMA and anatomical characteristics of leaf mesophyll structure.

		LMA (g m^{-2})	LNC (g m^{-2})	Leaf thickness (μm)	Mesophyll cell area (μm^2)	Mesophyll cell number/L (/ μm)	Mesophyll cell occupancy
Nipponbare	low	46.86	1.08	108 \pm 7 ns	335 \pm 42 ns	179 \pm 22 abc	60.0 \pm .5 ns
	high	53.47	2.13	124 \pm 15	251 \pm 50	165 \pm 4 c	58.2 \pm 2.7
Takanari	low	53.12	1.03	112 \pm 9	252 \pm 21	150 \pm 13 c	56.2 \pm 2.8
	high	57.06	2.46	113 \pm 8	251 \pm 57	194 \pm 32 bc	57.8 \pm .5
Shennong265	low	51.94	1.52	124 \pm 10	328 \pm 34	167 \pm 19 ab	59.3 \pm 3.7
	high	62.74	2.83	114 \pm 5	309 \pm 45	221 \pm 22 a	61.5 \pm .5
Cultivar (C)		—	—	NS	NS	NS	NS
Nitrogen fertilizer level (N)		—	—	NS	NS	*	NS
C \times N		—	—	*	NS	*	NS

Notes. Values are means \pm SD for $n = 3$. Values followed by the same letters indicate no significant difference among cultivars or N treatments at $p < .05$ (Tukey's test). A two-way ANOVA was conducted to evaluate the effects of cultivar (C), nitrogen fertilizer level (N), and their interactions (C \times N) on each leaf anatomical characteristic for the pot experiment.

*Significant at $p < .05$ and NS indicates non-significance.

in the same treatment, and that of Shennong265 in the low-N treatment was 26% higher than that of Nipponbare in the same treatment. In Takanari and Shennong265, however, the ratio of P_n/C_i to LNC in the high-N treatment was lower than that of Nipponbare, despite no difference in closely correlated with leaf $P_n/C_i/LNC$ among three cultivars in the low-N treatment (Figure 4).

The number of mesophyll cells between the two small vascular bundles (CN/L) was significantly greater in Shennong265 in the high-N treatment. There were no significant differences, however, in leaf thickness, cell area, length between small vascular bundles, or mesophyll cell

occupancy (Figure 5; Table 4). Although the effect of cultivars on CN/L was not significant, the effects of N fertilizer levels and their interactions on CN/L were significant ($p < .05$; Table 4).

Discussion

In the present study, we found that Shennong265 had higher P_n than Takanari and Nipponbare under various N availabilities. The P_n value of C_3 plants is limited by either the capacity of RuBP carboxylation or that of RuBP regeneration, and the limitation varies with CO_2 concentration

(Farquhar et al., 1980). In other words, the limiting step of P_n is influenced by the balance of maximum rate of Rubisco activity (V_{max}), potential rate of electron transport (J_{max}), and CO_2 concentration in the chloroplast (Hikosaka et al., 2006). Rubisco concentration is closely related to V_{max} and leaf Rubisco content is a key factor of the limitation of CO_2 assimilation (Farquhar et al., 1980). LNC is closely correlated with leaf Rubisco content, and one of the most important parameters on photosynthesis (Evans, 1989; Hirasawa et al., 2010; Makino et al., 1992). There are significant varietal differences in LNC in rice cultivars even under the same N fertilizer treatment (Hirasawa et al., 2010; Kanemura et al., 2007). LNC of Shennong265 was higher than that of Nipponbare or Takanari at the same rate of N application (Table 2, 4). This was mainly caused by high LMA of Shennong265 (Table 4). The initial slope of P_n - C_i curve, the ratio of P_n/C_i , can be often used instead of V_{max} . The ratio of P_n/C_i was the highest in Shennong265 in low-N and high-N treatments in both pot and field experiments (Table 2; Figure 3). Thus, the high leaf photosynthetic capacity of Shennong265 can be explained by high LNC and P_n/C_i .

High LNC and Rubisco content of Shennong265 could be correlated with the well-developed mesophyll anatomy, such as the large value of CN/L (Figure 5; Table 4). According to previous studies, the high photosynthesis allele of *GPS* (*GREEN FOR PHOTOSYNTHESIS*), contributing to the high leaf photosynthetic capacity of Takanari, was found to be a partial loss-of-function allele of *NAL1* (*NARROW LEAF1*). This allele increases the number of mesophyll cells between the small vascular bundles and also thickens the leaves (Takai et al., 2013). Similarly, CN/L and high LNC were suggested to be the major causes of high P_n in Shennong265 (Figure 5; Table 4). There are also apparent characteristics in the morphological phenotype of Shennong265; the flag leaf is wider and shorter in Shennong265 (Jiang et al., 2010; Zhu et al., 2009). In addition, the LAI of Shennong265 is much lower than in non-EP, regardless the nitrogen availability at both panicle initiation stage and heading stage (Figure 2). Thus, Shennong265 seems to have a tendency to distribute more N to leaf mesophyll structure rather than to LAI expansion. Since LAI is one of the most important factors in agricultural studies (Soltani & Galeshi, 2002), the low LAI of Shennong265 should be a disadvantage in terms of dry matter production. By applying more N fertilizer or greater planting density, high photosynthetic capacity Shennong265 is expected to contribute to the further increase of the biomass production.

The value of P_n/C_i /LNC totally decreased as N fertilizer level increased (Figure 4), and it is suggested that the photosynthetic capacity was approaching to the saturation under the high N availability. There was varietal difference in the ratio of P_n/C_i to LNC among three cultivars (Table

3), and that of Shennong265 was likely to be lower compared with other cultivars (Figure 4). The value of P_n/C_i to LNC can be influenced by g_m (mesophyll conductance), Rubisco activity, or the ratio of leaf Rubisco content to LNC (Makino et al., 1984a, 1984b). In this *in vivo* study, all of these are the possible interpretations to explain the variation of P_n/C_i /LNC. Especially, the observed difference in the mesophyll structure suggests the significance of g_m (Adachi et al., 2013). The value of g_m has recently been recognized as one of the most important determinants of P_n , especially in C_3 plants (Makino, 2011; Terashima et al., 2011; Warren, 2008). Furthermore, two backcrossed inbred lines derived from Takanari and Koshihikari (an elite *japonica* variety) have extremely high P_n , well-developed lobes of mesophyll cells, and a greater g_m than Takanari (Adachi et al., 2013). Photosynthetic performance of Shennong265 might be further improved if it is combined with the better ratio of P_n/C_i to LNC seen in the other varieties examined in this study.

Greater LNC has been linked to high g_s on several crop species (Yamori et al., 2011), and the value of g_s of *indica* varieties was larger than that of *japonica* varieties (Maruyama & Tajima, 1990). The high P_n of Takanari, a high-yielding *japonica-indica* cross-variety, was due to high g_s (Taylaran et al., 2011). The value of g_s in Takanari was also much higher than that of Nipponbare or Shennong265, even under low-N treatment in the present study (Figure 3). Conversely, the value of g_s was lower in Shennong265. These findings suggest that P_n of Shennong265 is also possible to be improved by combining with high g_s derived from Takanari,

In conclusion, Shennong265 showed high leaf photosynthetic rate under various nitrogen availabilities. It was even higher than that of Takanari under some conditions. This phenotype was achieved by greater LNC and P_n/C_i , which may partly be explained by the mesophyll structure including the mesophyll cell number. Leaf photosynthetic capacity in Shennong265 could be further improved by introducing the greater stomatal conductance or higher P_n/C_i /LNC during future breeding.

Disclosure statement

No potential conflict of interest was reported by the authors.

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*In Japanese with English abstract.

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